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Investigation of seed quality and seed surface modification changes in black soybean (*Glycine max* L. Merr) affected by plasma activated solution

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Abstracts

Plasma-activated solution (PAS) is attractive due to the shorter process and no need gaseous phase. The objective was to find whether PAS can improve seed quality of black soybean cv. Sukhothai 3 by seed coat modification and to enquire optimum treatment times. PAS was generated by treating 0.05% H_2O_2 with UV-C radiation at 0.5 l min-1 flow rate to obtain PAS-H_2O_2. Treated seeds and untreated were determined physical properties, seed quality, microbial population, and morphology changes. The results revealed that seed germination and the number of normal seedlings treated by PAS-H_2O_2 were non-significantly higher than untreated samples. While seed vigor, root length, and seedling dry weight showed the maximum level when subjected to the PAS-H_2O_2 condition for 5 minutes. The exposure duration of PAS-H_2O_2 treatment did not affect seedling stem development. The PAS-H_2O_2 could control total microorganisms to a standard level and the colony was reduced. The seed coat features, the changes of cell expansion and the number and size of seed pores increased than control treatment. This study indicated that plasma-activated solution could improve soybean seed germination and seedling establishment by modifying seed coat properties cooperated with different effects of treating times.

Keywords: Plasma activated solution, Black soybean seed, Seed quality

1. Introduction

To enhance the seed germination rate and drought resistant seeds, the production could be improved [1]. However, their germination loss easily under unfavorable conditions especially high temperature and relative humidity [2, 3]. Some research showed that black soybean genotypes are more resistant to weathering than yellow ones [4]. They have been widely consumed in Asia countries due to their health benefits which are primarily associated with polyphenols usually anthocyanins, isoflavones, and phenolic acids which are proven to prevent some diseases [5]. Moreover, the most commonly used methods to enhance the seed germination are both physical and chemical methods such as magnetic field [6], high pressure [7], and gramma radiation [8].

Cold plasma technology, a pollution-free method, is interested in medicine (sterilization), and agriculture due to the numerous reactive oxygen and nitrogen species (RONS) which can be used to stimulate growth parameters, disease and stress resistance, and plant metabolism in various plants [9, 10]. Several works have already evidenced how a large variety of seeds treated with gaseous plasma usually cold atmospheric plasma (CAP) and plasma-activated water (PAW) can be expressed to accelerate seed germination and plant growth by modifying seed coat resulting seed permeability improved [11-13]. It induces poration, corrugation and hydrophilization of seed, leading to hydrophilic pores creation and water and reactive species absorption [12, 14]. Also, CAP creates pathogen-based plant disease therapy and by disrupting disease function and spores growth resulting in diseases resistance improve [10, 15]. However, CAP is still depended on working gas and RONS cannot be remotely and transported. PAW, indirect plasma treatment makes the technique attractive due to it can be stored and made remotely and transported. Despite significant recent progress in the development and optimization of CAP and PAW, their reactions cannot be occurred without dependence on a CAP device. An extensive use of CAP technology in modern agriculture in terms of final crop growth and disease resistance contribution should be further studied [13]. While, PAW technology have been tested for plasma fertilizer and disease defend more than pre-seeded treatment [16, 17]. Recently, PAS has become more interested technology due to it has the shortest process and does not require a gas phase. PAS is generated by exposing H_2O_2 solution with UV-C radiation to acquire plasma activation [18].The low concentration (3 x 10-5 $moll^{-1}$) of H_2O_2 from the reaction is a nontoxic, ecologically safe regulator for various plants growth [19]. Some research demonstrated using high concentration of H_2O_2 as a medium to obtain PAS-H_2O_2 could reduce insecticide in kale leaves [20] and decontaminate pathogen in others [15, 18]. Nevertheless, excessive of them could generate RONS resulting in reverse effects to cell plant, therefore the use in lower H_2O_2 acted as a significant motivation for both germination rate and plant length [21].

This study aimed to prove seed quality of black soybean after treatment through seed coat modification changes and seeks an optimum treatment for soybean seed enhancement.

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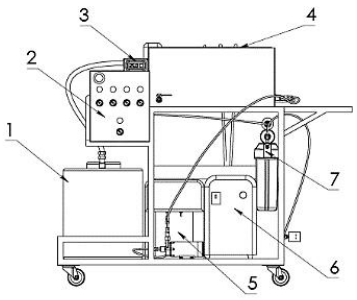
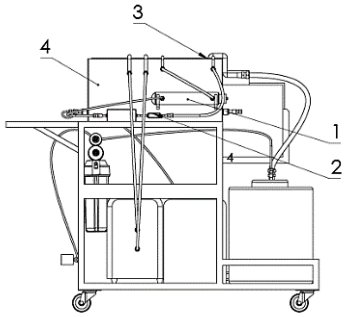
2. Materials and methods

2.1 Materials

The plasma-activated hydrogen peroxide solution generator (PAS-H_2O_2) is schematically illustrated in Figure 1 which was generated at Faculty of Engineering, Chiang Mai University. The main device consisted of UVC lamp (254 nm. Germicidal UVC), a nano-bubble (NB) generator, and a discharge chamber with a length of 20 cm, a width of 13 cm, a height of 10 cm, and a 1 inch thickness. Healthy uniform seeds of black soybean cv. Sukhothai 3 (ST.3) produced by the Chiang Mai Field Crops Research Center (CMFCRC), Chiangmai were stored in controlled-climate condition during experiment (temperature of $\leq$ 20 ^{o}C, 66-67 % RH).



(a) Front side 1. RO water tank 2. Control box 3. Temperature controller 4. Plasma chamber 5. Water cooler 6. NB generator 7. RO water filter



(b)Back side 1. UVC water sterilizer 2. Flow rate display 4. Water bath

Figure 1 A schematic diagram of PAS-H_2O_2 generator

2.2 Preparation of PAS-H_2O_2 solution

UVC lamp, a source of stimulating energy, exposed to electrolyzed oxidizing water (EO) mixed with 0.05 % H_2O_2 solution to generate PAS-H_2O_2 through the fluid circulating system with a constant flow rate of 0.5 $lmin^{-1}$ in different times of plasma discharge (1-15 min). During plasma treatment, the circulating cooling system was used to control constant temperature of 25$\pm$ 1 ^{o}C. The longevity of reactive species was made through NB generator. The schematic diagram of experiment set up is shown in Figure 2.

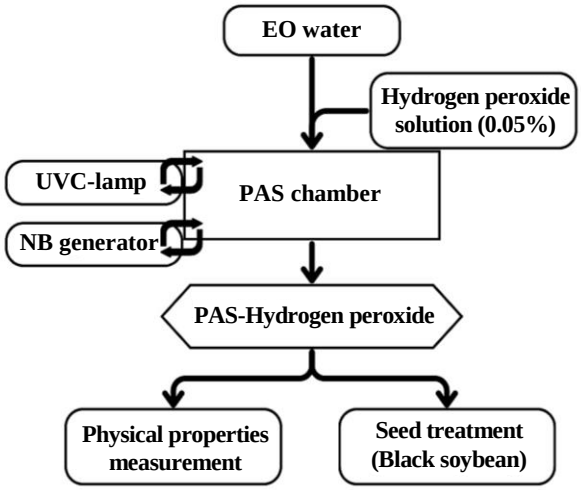


Figure 2 The schematic diagram of experiment setup**.**

2.3 Seed treatment

Soybean seeds were selected and soaked in PAS-H_2O_2 at 1, 5, 10, and 15 min. Seed samples were then dried and keep constant moisture content (12 %) using silica gel before analyzing seed quality. Untreated seeds were not submitted in plasma treatments. It was CRD with 4 replications and treatments were times of exposure.

2.4 Research data measurement

2.4.1 Measurement of the physical properties of PAS-H_2O_2

PAS-H_2O_2 was detected immediately oxidation-reduction potential (ORP), electrical conductivity (EC), and pH at different plasma activation times using ORP probe, EC meter, and pH meter, respectively. Also, dissolved oxygen (DO) was recorded by DO meter.

2.4.2 Determination of seed quality

Seed germination were analyzed by placing seeds between moist paper towels and kept in an incubator for 8 d at 25± 2 ºC with 94± 2 % RH. Normal seedlings were counted on day 8. Seed vigor was detected by AA-test by pre-conditioning seeds on the sieve in a jar containing water to obtain 100 % RH. Samples were then accelerated aging in a controlled temperature incubator for a period of 72 h. at 41ºC. Vigor was measured by germination percentage. Seedling growth were evaluated from BT-test and normal seedlings were detected average root and stem lengths on and then dried in the hot air oven for a period of 72 h. at 70 ºC for detecting seedling dry weight(SDW). The test were based to the ISTA rule [22].

2.4.3 Analysis of aerobic plate count

Total microorganisms were count by diluting samples and spread plating into media. Then, colonies were counted and calculated based on Bacteriological Analysis Manual [23] and Standard Methods for Food Analysis, Volume II [24].

2.4.4 Field emission scanning electron microscope for analyzing seed coat modification

Seed coat modification and morphology were determined by FE-SEM (Model: Clara, Tescan, Czech Republic) with a magnification of 1,500 times. Seeds were cut crosswise and lengthwise and were put on aluminum stub, coated with gold for 10 minutes before analysis.

3. Results

3.1 Physical properties of PAS-H_2O_2

Physical properties of PAS-H_2O_2 with different exposure times are given in Table 1. The ORP is an indicator of capability for oxidation in plasma and conductivity is an indicator of reactive species concentration in the PAS-H_2O_2. The ORP and conductivity values were higher than those of the control treatment and reduced as the time increased. In the same way, The DO value diminished with longer period. Conversely, the pH value and temperature expressed different results. The ORP and conductivity enhanced the best level when treated by PAS-H_2O_2 treatment for 1 and for 1 and 5 min., respectively.

Table 1 Physical properties of PAS-H_2O_2 and control treatments

Exposure time (min)	ORP (mV)	pH	EC (mS/cm)	DO (mg/L)	Temperature (ºC)
Control	140±1.12^{c}	7.86±0.00^{e}	2.00±0.05^{c}	0.87±0.00^{a}	25.0±0.00^{b}
PAS-H_2O_2-1	173+1.12^{a}	7.94±0.00^{d}	2.35±0.05^{ab}	0.86±0.00^{b}	25.6±0.00^{ab}
PAS-H_2O_2-5	163+1.12^{b}	7.95±0.00^{c}	2.44±0.05^{a}	0.74±0.00^{c}	25.8±0.00^{ab}
PAS-H_2O_2-10	151+1.12^{c}	7.98±0.00^{b}	2.25±0.05^{b}	0.65±0.00^{d}	26.1±0.00^{a}
PAS-H_2O_2-15	146+1.12^{d}	7.99±0.00^{a}	2.25±0.05^{b}	0.65±0.00^{d}	26.2±0.00^{a}

3.2 Effect of PAS-H_2O_2 on soybean seed quality

3.2.1 Seed germination rate and seed vigor

Black soybean seed germination cv. ST.3 (Sukhothai3) under PAS-H_2O_2 treatments are shown in Figure 3A. Even no differences were found among treatments they gave seed germination higher than those of the control. While, the maximum seed vigor were obtained from treating PAS-H_2O_2 for 1 and 5 min. (Figure 3B). Finally, the proper time for maximizing both seed germination, and seed vigor were exposing for 1 and 5 min. that could be increased by 3.2 % when compared to the control.

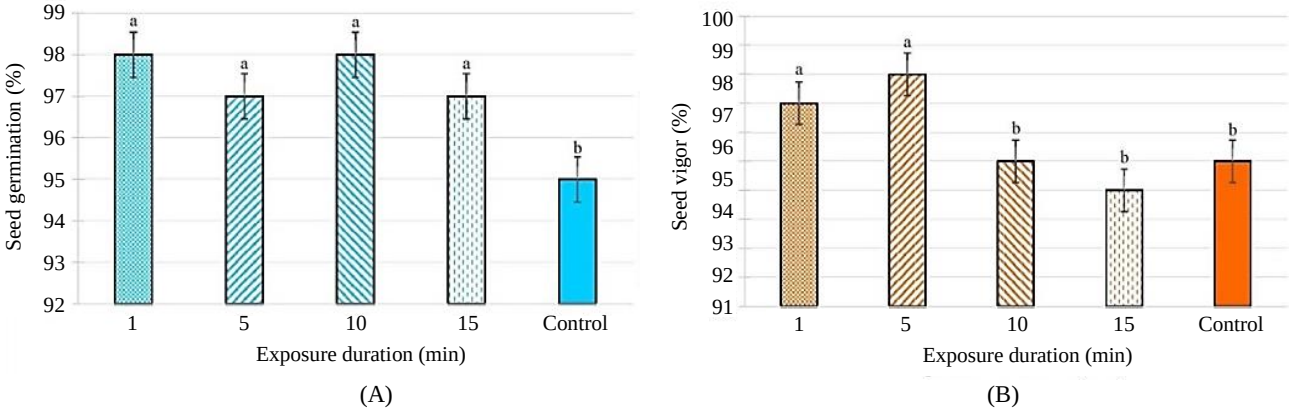


Figure 3 Seed germination (A) and seed vigor (B) of black soybean as affected by PAS-H_2O_2

3.2.2 Seedling growth

Under PAS-H_2O_2 influence, number of normal seedlings of all treatments raised and they were higher than those of control treatment. Although, stem length of all except treating for 15 min. did not differ from the control their grew up as time extended and moderately diminished when exposed for 15 min. (Table 2 and 3). Also, the maximum SDW was obtained from treating for 5 min. No significant differences in SDW were found among during exposure times of 1, 10, 15 min. and the control. Over all, the maximize number of normal seedlings, root and stem lengths include SDW were obtained to treating for 5 min. which could be raised by 8.9, 20.2, 6.8, and 12.5 % when compared to control, respectively.

Table 2 Root length and stem length of black soybean as affected by PAS-H_2O_2

Exposure time (min)	Root length (cm)	Stem length (cm)
Control	14.82±0.50 ^{cd}	14.02 ±0.53 ^{a}
PAS-H_2O_2-1	16.31±0.50 ^{b}	14.33 ±0.53 ^{a}
PAS-H_2O_2-5	17.81±0.50 ^{a}	14.97 ±0.53 ^{a}
PAS-H_2O_2-10	17.03±0.50 ^{ab}	15.08 ±0.53 ^{a}
PAS-H_2O_2-15	13.66±0.50 ^{d}	11.95 ±0.53 ^{b}

Table 3 Number of normal seedling and SDW of black soybean as affected by PAS-H_2O_2

Exposure time (min)	No of.normal seedling (%)	Seedling dry weight (SDW) (g/plant)
Control	90 ±0.60 ^{b}	0.24 ±0.00 ^{b}
PAS-H_2O_2-1	100 ±0.60 ^{a}	0.24 ±0.00 ^{b}
PAS-H_2O_2-5	98 ±0.60 ^{a}	0.27 ±0.00 ^{a}
PAS-H_2O_2-10	98 ±0.60 ^{a}	0.23 ±0.00 ^{b}
PAS-H_2O_2-15	96 ±0.60 ^{a}	0.24 ±0.00 ^{b}

3.3 Effect of PAS-H_2O_2 on seed surface fungal contamination

Table 4 and Figure 4 are displayed the colonies of microorganisms in soybean seed coat. In a comparison to the control, all treated seeds with PAS-H_2O_2 exhibited the lower of them Moreover, the use of all times of plasma discharge could reduce total viable count between 46-95 % depend on exposure time raised.

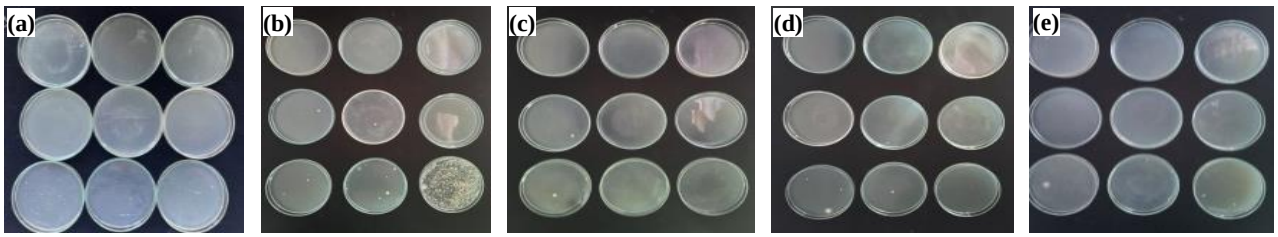


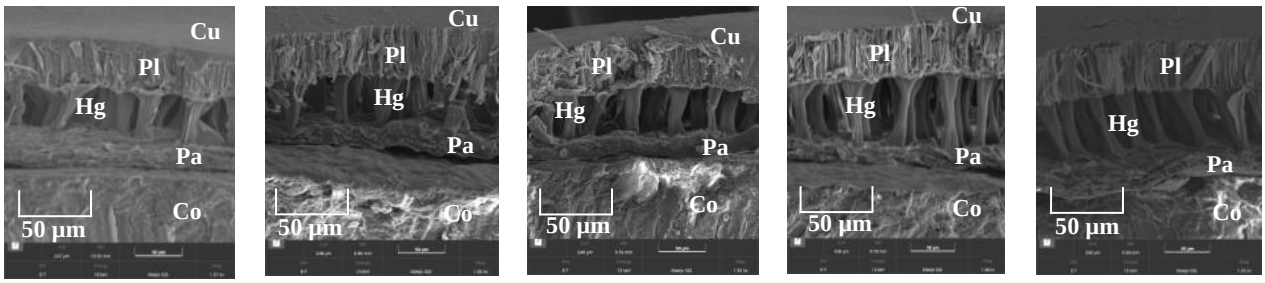
Figure 4 The colonies of microorganisms in soybean seed coat as affected by PAS-H_2O_2 at different times of exposure; (a) Control, (b) 1 min., (c) 5 min., (d) 10 min., (e) 15 min.

Table 4 Total microorganisms in black soybean seed surface as affected by PAS-H_2O_2

Exposure time (min)	Total microorganisms (%)
Control	100 ±0.30 ^{a}
PAS-H_2O_2-1	54 ±0.30 ^{b}
PAS-H_2O_2-5	12±0.30 ^{cd}
PAS-H_2O_2-10	5 ±0.30 ^{d}
PAS-H_2O_2-15	0 ±0.30 ^{e}

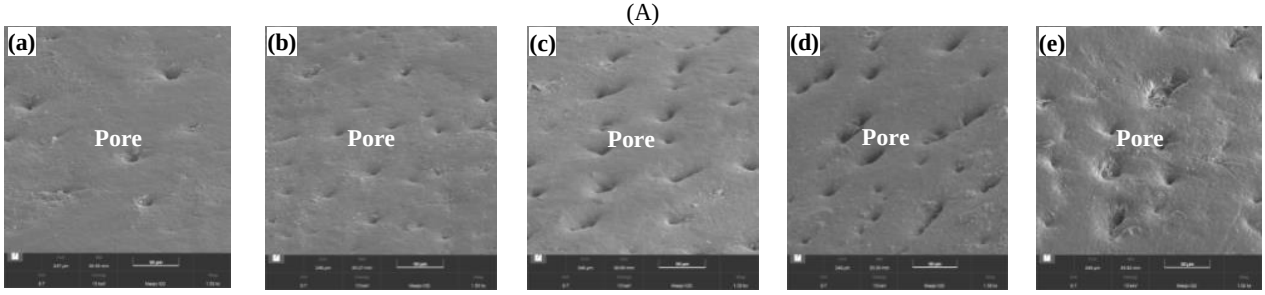
3.4 Seed coat modification changes as affected by PAS-H_2O_2

The apparent of seed coat structure treated by PAS-H_2O_2 is displayed in Figure 5A and 5B. The FE-SEM micrographs of seed coat patterns showed cell enlargement especially in hourglass cells (Hg) resulting in compression of parenchyma cells. Their elongation increased as time extended and treating for 15 min. acted the most of all. In comparison to untreated seeds, seed coat surface expansion after PAS-H_2O_2 treatment also conspicuously expressed. The number of seed pores enhanced and their size gave bigger than those of control and they did more larger as time extended. PAS-H_2O_2 could expand seed pore and size ranged of 53.8-92.3 and 7.1-24.5% depend on plasma time increased, respectively (Table 5).



Cu = Cuticle, Pl=Palisade cells, Hg=Hourglass cells, Co=Cotyledon, Pa = Parenchyma cells

(A)



(B)

Figure 5 A FE-SEM image of seed coat structural features (A) and seed coat surface (B) of soybean treated by PAS-H_2O_2 at different times of exposure; (a) Control, (b) 1 min., (c) 5 min.,(d) 10 min., (e) 15 min.

Table 5 Pore diameter and number of pore in seed surface as affected by PAS-H_2O_2

Exposure time (min)	Pore number(µm)	Number of pore(pore/10,000 µm^2)
Control	9.8 $\pm$0.21 ^{c}	2.5 $\pm$0.10 ^{d}
PAS-H_2O_2-1	10.5 $\pm$0.21 ^{b}	4.0 $\pm$0.10 ^{b}
PAS-H_2O_2-5	10.6 $\pm$0.21 ^{b}	4.4 $\pm$0.10 ^{b}
PAS-H_2O_2-10	10.9 $\pm$0.21 ^{b}	4.7 $\pm$0.10 ^{b}
PAS-H_2O_2-15	12.2 $\pm$0.21 ^{a}	5.0 $\pm$0.10 ^{a}

4. Discussion

4.1 Physical properties of PAS-H_2O_2

It was noticed pH of all treatments showed alkaline values and raised as time expanded which were opposite from CAP research, however some research using PAW for growth parameter expressed the same results with this study [16, 25]. Giri et al. [26] reported enhanced efficiency of UV/ H_2O_2 oxidation at alkaline pH region is attributed to OH^- formation. Also, an increase of pH due to decomposition of H_2O_2 into OH^-, water, and oxygen therefore a suitable H_2O_2 dosage exhibited enhanced performance of plasma activation [27] should be further investigated. While, the conductivity raised when the pH slightly dropped due to the higher mobility of H^+ relates to OH^- [28]. Also, a slight increase in pH when the time is extended might be caused by the temperature of solution described by Judee et al. [29] and Barron et al. [30]. They reported the pH was distorted by higher temperature of solution leading to a reduction in its viscosity and therefore an increase in its ion mobility and overall ion concentration. However, it should be further study in this issue for more explanation.

The introduction of other ROS such as superoxide anion ($O_2^{\cdot-}$**),** H_2O_2, and singlet oxygen ($^1O_2$), ions and oxygen in solution might be responsible for DO enhancement in PAS-H_2O_2 [28]. Furthermore, ORP in this study had lower than other reports. Previous works have evidenced how to generate large amount of RONS to control microorganism and reduce pesticide from plants. [15, 18, 20]. The higher ORP it gets, the higher in RONS it generates. Nevertheless, excessive ORP could be reverse effects to protein and cell of plants since plenty of RONS. Therefore, some research concluded lower in H_2O_2, acted as a significant motivation for both germination rate and plant length [21].

4.2 Effect of PAS-H_2O_2 on soybean seed quality

4.2.1 Seed germination rate and seed vigor

The reasons why seeds treated by PAS-H_2O_2 increased seed germination and seed vigor are first described by the role of RONS on seed surface modification, growth parameter, disease and stress resistance and metabolism [10]. The second one is explained by Zhou et al. [21] and Andreev et al. [19] who concluded higher ORP could generate more ROS than lower ORP, leading to a more significant effect on seed quality.

4.2.2 Seedling growth

The results of this study were Similar to Ahmad et al. [18] demonstrated that PAS-5% H_2O_2 could reduce seed infection and improve seed quality in pepper.

4.2.3 Effect of PAS-H_2O_2 on seed surface fungal contamination

Several reports supported this research such gaseous plasma have demonstrated the decontamination of fungi especially *A. flavus* and *A.* spp. and extended treatment time must be used to inactivate fungi that are depended on their species [31-33]. Also, Rathor et al. [17] who found PAW could eliminate pathogens and microorganisms. Similar results were obtained by Ahmad et al. [18] who concluded using PAS-5% H_2O_2 could decontaminate seed infection of pepper.

4.2.4 Seed coat modification changes as affected by H_2O_2 PAS

The seed coat structural changes after PAS-H_2O_2 treatment could be described by the theory of plasma etching effects on seed coat increased the hydrophilicity of seeds, activity of hydrolytic enzyme leading to hydrophilic pores creation and water and reactive species absorption [12, 14]. Reactive species especially OH^- and H_2O_2 have an important role in seed germination in terms of an etching effect [21, 34].

5. Conclusions

The use of PAS-0.05% H_2O_2 for 5 min. had the optimum condition for soybean seed quality. It gave the pretty ORP and the highest EC which was suitable for both oxidation reaction and RONS concentration usually OH^- and H_2O_2 for availability and their movement. Seed coat modification induction expressed by cell enlargement and the changes of seed pores leading to a hydrophilization of seed surface and subsequently seed quality enhancement. In order to be useful for industrial scale with a focus on universality and low production cost, pre-seeded treatment with PAS-0.05% H_2O_2 could apply for 1-4 min. To further broaden potential application, it should consider the objectives of soybean utilization (seed, food and raw materials), conditions, and the defect. In case of soybean seed enhancement, rapid loss of seed viability, applying low H_2O_2 as a substrate of the solution, with the appropriate time of plasma discharge is the proper method. It expresses in faster seed germination, higher seed vigor and seedling growth development include microorganisms inactivation. Moreover, avoiding from an excessive H_2O_2 and or longer period of plasma discharge, they might be reverse effects to protein and cell of soybean. It should be further investigated the response of soybean with different level of preliminary seed quality to plasma solution that cv. Sukhothai 3 is the medium seed quality in which pretty obviously response to plasma solution.

6. Acknowledgements

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